Reference: *Biol. Bull.* **187:** 300–303. (December, 1994)

High-Resolution Measurement of the Time Course of Calcium-Concentration Microdomains at Squid Presynaptic Terminals

MUTSUYUKI SUGIMORI[1,3], ERIC J. LANG[1,3], ROBERT B. SILVER[2,3], AND RODOLFO LLINÁS[1,3]

[1]*Department of Physiology and Biophysics, New York University Medical Center, 550 First Avenue, New York, New York 10016,* [2]*Section and Department of Physiology, Cornell University, Ithaca, New York 14853-6401, and* [3]*Marine Biological Laboratory, Woods Hole, Massachusetts 02543*

Transmitter release is considered to be a secretory event triggered by localized calcium influx which, by binding to a low-affinity Ca^{2+} site at the presynaptic active zone, initiates vesicular exocytosis (1–7). In previous experiments with aequorin-loaded presynaptic terminals we visualized, upon tetanic presynaptic stimulation, small points of light produced by calcium concentration microdomains of about 300 μM (5). These microdomains had a diameter of about 0.5 μm (5) and covered 5–10% of the total presynaptic membrane with an average density of 8.4 μm^2 per 100 μm^2, corresponding closely to the size and distribution of the active zones in that junction (6, 7).

To understand in more detail the nature of these concentration microdomains, we obtained rapid video images (4000/s) after injecting the photoprotein n-aequorin-J into the presynaptic terminals of squid giant synapses. Using that experimental approach, we determined that microdomains evoked by presynaptic spike activation had a duration of about 800 μs. Spontaneous quantum emission domains (QEDs) observed at about the same locations as the microdomains were smaller in amplitude, shorter in duration, and less frequent.

These results illustrate the time course of the calcium concentration profiles responsible for transmitter release. Their extremely short duration compares closely with that of calcium current flow during a presynaptic action potential and indicates that, as theorized in the past (6–8), intracellular calcium concentration at the active zone remains high only for the duration of transmembrane calcium flow.

Received 20 September 1994; accepted 5 October 1994.

The spontaneous quantum emission domains (QEDs) observed prior to stimulation were imaged over an area corresponding to ≈20% of the total active zone of the preterminal studied. They occurred at a very low rate (10–15/s) and were generally located at the same sites as the evoked microdomains (Fig. 1B).

Calcium entry following tetanic stimulation of the presynaptic axon (500 ms or 1 s at 100 Hz) was characterized by the appearance of calcium microdomain sites that appeared to blink on and off during the tetanic stimulation (Fig. 1). The localization of the evoked microdomains in the presynaptic terminal is shown in Fig. 1A, and as previously reported (5), double-labeling of the presynaptic and postsynaptic fibers with fluorescent dyes demonstrated that QEDs are localized at the active zones (9).

These results suggest that the spontaneous and evoked light emissions probably correspond to spontaneous (10, 11) and voltage-evoked calcium channel openings, respectively. The low frequency of such light points indicates, however, that only a very small percentage of the total number of calcium entry events are being observed. Indeed, for spontaneous release given calculated levels of ≈35,000 quanta/s for the total active zone (10, 11), the area observed (20%) should generate ≈7000 QEDs/s rather than the 10–15 observed if we assume that one activated zone releases one QED. This number is based on the assumptions that (1) every spontaneous event is triggered by calcium entry at the active zone (which will overestimate the QEDs because some spontaneous transmitter release is calcium independent), and (2) each calcium entry event activates

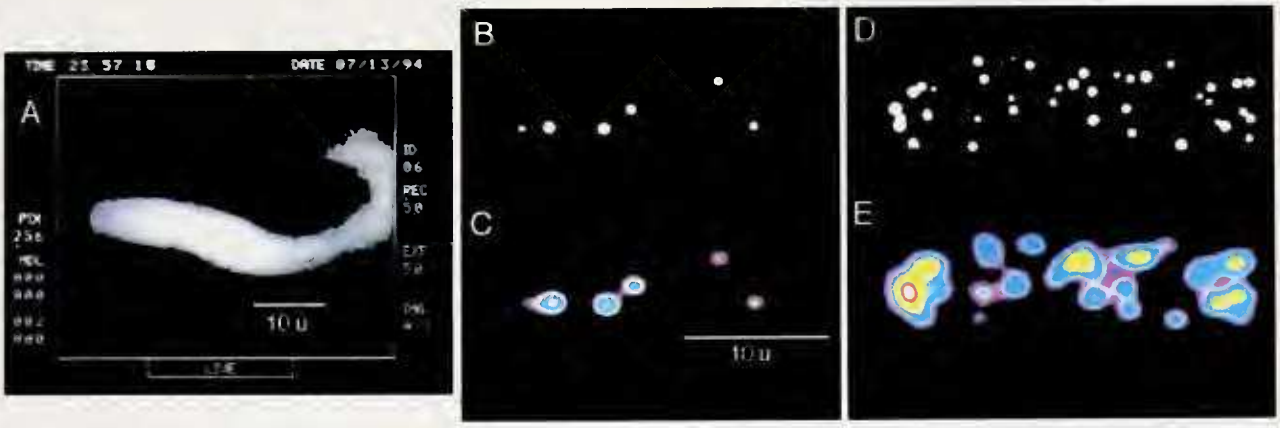

Figure 1. Fluorescence image and light emission from a squid synaptic preterminal injected with *n*-aequorin-J.

A hybrid synthetic aequorin, *n*-aequorin-J, with a sensitivity to $[Ca^{2+}]$ on the order of 10^{-4} *M*, developed by Shimomura, Musicki, and Kishi (19) was used as an $[Ca^{2+}]_i$ indicator. This photoprotein was chosen because its low binding constant requires relatively large calcium concentrations to activate photon emission ($\approx$300–400 μM [10]), which is triggered with a short delay ($\approx$750 s [12]). In addition, it has the advantage of not substantially modifying the buffering properties of the intracellular milieu.

Presynaptic injection of this aequorin (5) in the squid (*Loligo pealii*) giant synapse (20), allowed the selective detection of high-calcium-concentration microdomains (5). The distribution of the injected aequorin inside the presynaptic terminal was determined by visualizing a fluorophore (rhodamine-labeled Dextran) that was injected with the photoprotein (5). The diffusion profile was visualized using a fluorescence microscope with a 40× water-immersion objective lens (N.A. 0.75). Aequorin luminescence was measured with a dMCP intensified camera operated in the photon-counting mode (12). The images were stored on conventional videotape (30 video frames/s), a fast-tape system (NAC HSV-400) with a 5-ms time resolution, or a digital system (Kodak HSC) with a 250-μs resolution (12). The synapses were dissected under flowing seawater, and during the experiments the preparation was bathed in artificial seawater containing 10 mM Ca^{2+}.

(A) Fluorescence image showing the preterminal on the larger of the post axons (not visible). (B) Spontaneous light emission recorded over 500 ms. (C) Same as in B, showing the light intensity (relative scale [12]) in pseudocolor. (D and E) Same as in B and C but taken over a 500-ms period of tetanic stimulation at 100 Hz.

aequorin (which also overestimates because such reactions—being dependent on concentration and time—are far from fully efficient).

The QED frequency for evoked release occurred at a rate of 250–500/s, with superimposed QEDs occurring at a rate of 3 to 5/s. QED frequency for evoked release was 20 to 30 times greater than the QED frequency for spontaneous activity (Fig. 1D and E). The QED frequency for the relative increase in evoked release was at about the level expected assuming a quantal content of 5 to 10 $\times$ 10^3 per action potential (3, 11). With 1 s of tetanic stimulation of 100 Hz, the quantal release would be near 0.5 to 1 $\times$ 10^6.

The experimental results indicate detection levels three orders of magnitude smaller than expected from such calculations. Two possible explanations for this very low yield are (1) that QEDs are generated only when calcium channel openings occur in particular spatial distribution (*e.g.*, clustering), *i.e.*, the so-called neighbor effect (8); (2) that our detection system sees only a small percentage of the photons emitted (12). Preliminary measurements suggest that only 0.22% of the light emitted is captured by the present system (12), which supports the view that the low observed QED yield is due to technical limitations in detection (12).

To compare the time course for evoked QEDs with that of the presynaptic calcium current triggered by a simulated action potential, these events were superimposed on the same time base (Fig. 2). The time course for I_{Ca} superimposes adequately on the time course of a microdomain evoked during the activation of similar presynaptic spikes (Fig. 2B). Note, however, that the light emission seems to outlast that of the transmembrane current. This is to be expected because high-level light emission does leave an afterglow in the photosensors (12). Indeed, as shown in Figure 2 panel C, the time course for the microdomain corrected for this distortion (12) matches quite closely the time course of the macroscopic I_{Ca}.

The results presented here demonstrate for the first time the true time course for the calcium microdomain at a chemical presynaptic terminal. These findings are in agreement with the time course for spike-evoked calcium currents (13) as well as with model results (8) for

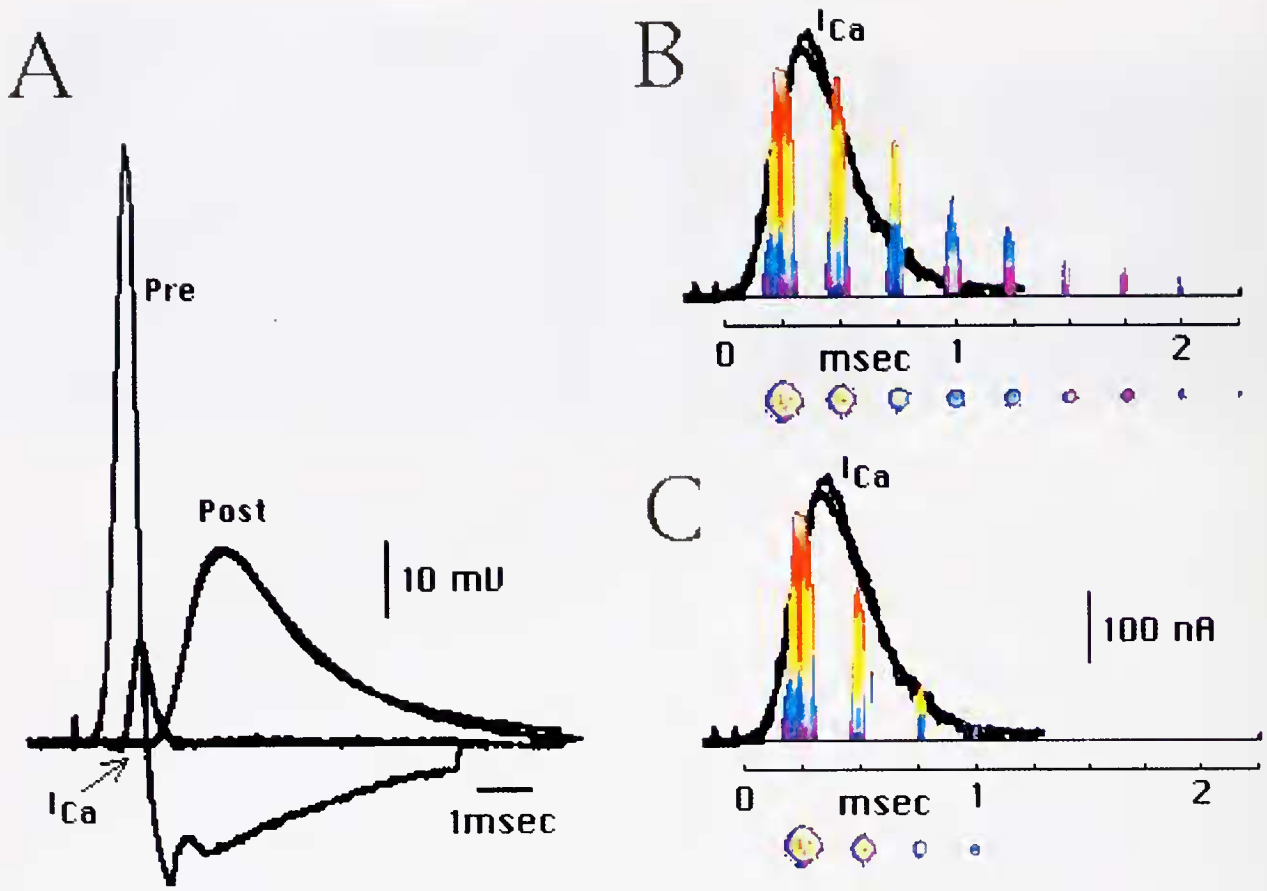

Figure 2. Time course for evoked calcium concentration microdomains. (A) Illustration of the time course for a presynaptic action potential (Pre) that simulates the voltage profile of a presynaptic spike recorded from the same fiber, before block of sodium and potassium channels (from 7); the calcium current (I_{Ca}) generated by the transient depolarization; and the postsynaptic response (Post) triggered by I_{Ca}. The presynaptic current (ICa—arrow), basically a tail current with an overall duration of $\approx$800 μs, and the postsynaptic potential obtained in previous experiments (7) are shown. (B and C) Superimposition of the time course for the calcium microdomain (amplitude on a relative scale) and the time course for the presynaptic calcium current. (B) Amplitude of raw light measurements taken every 250 μs. (C) Amplitudes after correction for afterglow. The area of the color circles under each time point relates to the amplitude of the light emission during that time bin.

the distribution and time course for transient calcium-concentration profiles, given by the equation for the analytical solution for three-dimensional calcium diffusion:

$$C(r,t) = \frac{2.0 \times 10^3 \times J}{4.0 \times \pi \times FAR \times Dca \times r} \, erfc\left(\frac{4}{2.0\sqrt{Dca \times t}}\right)$$

where C (r, t) is the calcium concentration at time, t, and distance, r, from the channel; Dca is the diffusion coefficient for calcium; FAR is the Faraday constant; J is the flux per channel; and *erfc* is the complementary error function. This equation was used to calculate the calcium-concentration profile, which reaches a steady-state level of $\approx$300 μM at 500 Å from the channel pore in 1 μs and, following channel closure, decays to a level of 10 μM in a similar period for the same distance (8). Given the sensitivity level of *n*-aequorin-J, our results are, indeed, consistent with the model.

The time course reported here is different from that observed in other secretory systems such as the chromaffin cell (14), where the transient develops over 10 to 100 ms and where the calcium buffering properties may be quite different. However, our results are similar to the calcium concentration profile in individual frog-muscle sarcomeres following the activation of action potentials (14).

The overall duration of the microdomain is in agreement with the increased probability of release produced by a presynaptic spike. The calcium current produced by a presynaptic action potential has a peak at 0.3 ms and an overall

duration of ≈0.8 ms (13), very much in agreement with measurements observed with the present technique.

The observations described in this paper constitute a direct demonstration of the time course for the calcium concentration microprofiles at a presynaptic terminal. This time course matches closely that of the release process (13) and the average opening time for P-type calcium channels (15) which may be responsible for the presynaptic calcium current in this terminal (16). The results further indicate that the temporal coherence for transmitter release is indeed produced by a well-matched relationship between the kinetic properties for the opening of calcium channels and the duration of the presynaptic action potential (13).

These results confirm our previous conclusion that synaptic neurotransmitter release is triggered by high-calcium-concentration microprofiles at the active zone that induce a rapid triggering of vesicle fusion lasting for the duration of the calcium profile (3, 17). Since the time course of the evoked microdomain is about three times as long and about fivefold larger than that produced by spontaneous QEDs, the evoked release may be triggered by the opening of many more calcium channels per active zone than the spontaneous events.

The number of calcium ions that are detected in an evoked microdomain may be estimated as follows. Consider that a presynaptic action potential generates an I_{Ca} of about 300 nA (10, 13), a current of 0.5 pA/channel, and that a single channel allows the flow of about 150 to 200 calcium ions (7, 18), then $\approx 6 \times 10^5$ channels are opened to release 5000 to 10,000 vesicles (4, 11) or ≈15,000 calcium ions/vesicle (13). [This is in contrast to the estimated minimum of 200 ions/vesicle (one calcium channel) for the ciliary ganglion (18)]. Given that the number of active zones in a presynaptic terminal is about 5000 to 10,000 (6), the number of channels open per action potential for each active zone is ≈100. If single channels allow the flow of 150 to 200 calcium ions (7, 18), an evoked microdomain, using *n*-aequorin-J and the present imaging technique, may represent an influx of calcium of about 15,000 ions.

Acknowledgments

We thank Drs. O. Shimomura, S. Inouye, B. Musicki, and Y. Kishi for the generous gift of the custom aequorin preparations used in this study. We are also grateful to Mr. P. W. DelGrego (Motion System Analysis Division, Eastman Kodak Co.) for assistance with the digital high-speed video observations. Work supported by the N.I.H. (NS13742 to R.L.), the U.S. Air Force (F49620-92-J-0363 to R.L.) and the N.S.F. (DCB-9005343 and DCB-9308024 to R.B.S.).

Literature Cited

1. **Augustine, G. J., E. M. Adler, and M. P. Charlton. 1991.** The calcium signal for transmitter secretion from presynaptic nerve terminals. *Annals NY Acad Sci.* **635:** 365–381.
2. **Katz, B. and R. Miledi. 1967.** A study of synaptic transmission in the absence of nerve impulses. *J Physiol. (Lond.)* **192:** 407–436.
3. **Llinás, R. 1977.** Calcium and transmitter release in squid synapse. Pp. 139–160 in *Society for Neuroscience Symposia,* Vol. 2, W. W. Cowan and J. A. Ferendelli, eds. Society for Neuroscience, Bethesda, MD.
4. **Llinás, R., I. Z. Steinberg, and K. Walton. 1981.** Relationship between presynaptic calcium current and postsynaptic potential in squid giant synapse. *Biophys J* **33:** 323–352.
5. **Llinás, R., M. Sugimori, and R. B. Silver. 1992.** Microdomains of high calcium concentration in a presynaptic terminal. *Science* **256:** 677–679.
6. **Pumplin, E. F., and T. S. Reese. 1978.** Membrane ultrastructure of the giant synapse of the squid *Loligo pealei. Neuroscience* **3:** 685–696.
7. **Pumplin, E. F., T. S. Reese, and R. Llinás. 1981.** Are the presynaptic membrane particles the calcium channels? *Proc. Natl. Acad. Sci. USA* **78:** 7210–7213.
8. **Simon, S. M. and R. Llinás. 1985.** Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. *Biophys. J.* **48:** 485–498.
9. **Llinás, R., M. Sugimori, and R. B. Silver. 1994.** Localization of calcium concentration microdomains at the active zone in the squid giant synapse. Pp. 133–136 in *Molecular and Cellular Mechanisms of Neurotransmitter Release.* L. Stjärne *et al.,* eds. Raven Press Ltd., New York.
10. **Lin, J.-W., M. Sugimori, R. Llinás, T. McGuinness, and P. Greengard. 1990.** Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. *Proc Natl Acad Sci USA* **87:** 8257–8261.
11. **Miledi, R. 1967.** Spontaneous synaptic potentials and quantal release of transmitter in the stellate ganglion of the squid. *J Physiol. (Lond.)* **192:** 379–406.
12. **Silver, R. B., M. Sugimori, E. J. Lang, and R. Llinás. 1994.** Time-resolved imaging of Ca^{2+}-dependent aequorin luminescence of microdomains and QEDs. *Biol. Bull.* **187:** 293–299.
13. **Llinás, R., M. Sugimori, and S. M. Simon. 1982.** Transmission by presynaptic spike-like depolarization in the squid giant synapse. *Proc. Natl. Acad. Sci. USA* **79:** 2415–2419.
14. **Monck, J. R., I. M. Robinson, A. L. Escobar, J. L. Vergara, and J. M. Fernandez. 1994.** Pulsed laser imaging of rapid Ca^{2+} gradients in excitable cells. *Biophys. J* **67:** 505–514.
15. **Usowicz, M. M., M. Sugimori, B. Cherksey, and R. Llinás. 1992.** P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. *Neuron* **9:** 1185–1199.
16. **Llinás, R., M. Sugimori, J. W. Lin, and B. Cherksey. 1989.** Blocking and isolation of calcium channel from neurons in mammals and cephalopods utilizing a toxin fraction (FTX) from funnel-web spider poison. *Proc. Natl. Acad. Sci. USA* **86:** 1689–1693.
17. **Llinás, R., I. Z. Steinberg, and K. Walton. 1981.** Presynaptic calcium currents in squid giant synapse. *Biophys. J.* **33:** 289–322.
18. **Stanley, E. F. 1993.** Single calcium channels and acetylcholine release at a presynaptic nerve terminal. *Neuron* **11:** 1007–1011.
19. **Shimomura, O., B. Musicki, and Y. Kishi. 1989.** Semi-synthetic aequorins with improved sensitivity to Ca^{2+} ions. *Biochem. J.* **261:** 913–920.
20. **Young, J. Z. 1939.** Fused neurons and synaptic contacts in the giant nerve fibers of cephalopods. *Phil. Trans. R. Soc Ser B* **229:** 465–505.